Genetic Distance as a Function of Geographic Distance in Ohio Dusky Salamanders

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Abstract

Evolution typically begins as geographic separation, leading to genetic isolation, or speciation. 'Island examples' of geographic isolation are common, but situations less extreme lead to evolution. Mobility and degree of ubiquity influence geographic separation.

Northern dusky salamanders (*Desmognathus fuscus*) are neither mobile nor widespread. They remain within metres of streams because of moisture needs due to cutaneous respiration. Considering this, small amounts of geographic separation might drive evolution.

The purpose of this study is to investigate the degree of genetic similarity between two populations of dusky salamanders in Ohio. We expect greater differences between the sites, given their degree of geographic separation, than we do within the sites.

We collected DNA from dusky salamanders in two Ohio locations. Analysis included viewing of rehybridized DNA, and fragment length matching. We found small but significant genetic differences between the two populations.

Introduction

The concept of home is not unique to humans, and the idea that 'home is where the heart lies' is common to an endless variety of organisms (Part, 1994; Stepien and Faber, 1998; Pomeroy et al., 2000). This should be no surprise considering the importance of an individual's habitat to that individual's chances at health, survival, and reproductive success. Habitat plays a key role in interacting with an individual's genetic inheritance to determine the individual's fitness (Darwin, 1859). Appropriate habitat obviously allows for better fitness of an individual by providing for the nutritional and other requirements of that individual better than sub-par habitat would. Habitat may also relate to more effectively attracting mates of better quality and in larger numbers, depending upon the reproductive strategy of the species in question. Philopatry, returning to and mating in the area close to your own natal site, also has the possibility to have positive reproductive value (Part, 1994). Philopatry can also discourage extreme out-breeding, which may be deleterious to the fitness of offspring (Shields, 1982; Edmands and Timmerman, 2003). Many organisms have developed mechanisms by which they can limit extreme inbreeding as well as extreme out-breeding. However, philopatry may be the best way to limit extreme out-breeding, as it has the net effect of limiting contact with distantly related individuals,

for philopatric organisms (Starin, 2001; Pusey, 1996). The combination of these two groups of control mechanisms could allow for optimum out-breeding occurring. The idea of inbreeding as not necessarily bad is not new (Shields, 1982), in fact it is fairly well accepted that breeding with an individual of too much dissimilarity can lower one's reproductive fitness (Finke and Jetschke, 1999).

Humans have long known, at least anecdotally, that other animals have the ability to move, recognize a certain area as 'home,' and to return to that area after a displacement of considerable distance and for a length of time. There exist exhaustive accounts of animals finding their way back home through circumstances that we as humans find astounding. The routes of any migrating bird, salmon, eel, or any of numerous other organisms in their journey to and from home, are well known examples of long distance migrations. Even animals as relatively immobile, compared to these extreme examples, as snakes and frogs (Meakins and Al-Mohanna, 2003; Vasconcelos and Calhoun, 2004, respectively), have their own travels and typically manage to find their way back to their natal den or pond on schedule for many years.

What we have not known, however, pertains to the mobility of even smaller more fragile organisms, like terrestrial salamanders. Individual *Desmognathus fuscus,* one such animal, at a mere 8-10 centimetres total length (Karlin and Pfingsten, 1989), have been known to home for 50 metres or more (Juterbock, personal communication), or distances of over six-hundred times the length of the animal itself. When one considers the nature of *D. fuscus,* an ectothermic, nocturnal amphibian, (Orser and Shure, 1974) and generalist carnivore (Zug, 1993), this truly is a feat.

Such mobility allows for exchange of genetic information among individuals in a given area and potentially among individuals from limited geographic distance as well. By increasing the size of the potential gene pool of a group, several positive effects occur. This ability of members of a group to adjust to changing environmental conditions and to withstand predation and illness results in their success and that of their group. The reproductive success of varied individuals results in more variability within the group (Reed and Frankham, 2003).

Isolation (both initially geographic, and subsequently genetic) of a population, one of the necessary conditions for evolution (Dobzhansky, 1937), may be maintained by several mechanisms. The two major forms of isolation maintenance are pre-zygotic and post-zygotic isolation. Pre-zygotic isolation is the separation of two groups by mechanisms that prevent the initial union of sperm and egg to create a zygote, either by preventing mating from occurring (pre-mating) or by gamete incompatibility (post-mating) (Smith, 1966). Examples of pre-mating isolation include geographic isolation, incompatibility of genitalia, differences in mating seasons, and incompatibility of courting rituals. Pre-and post-mating isolation may be more of a driving

force in overall genetic isolation than post-zygotic isolation mechanisms (Servedio, 2001). Post-zygotic isolation is the separation of two groups by mechanisms that allow the formation of a zygote, but do not allow for the continuation of the hybrid individual. Examples include hybrid lethality, hybrid sterility, and lowered fitness of hybrid individuals, each of which can result in genetic isolation with varying rapidity (Hayashi and Kawata, 2001).

Geographic isolation, perhaps the most common reason for genetic divergence, often is described in one of its most severe forms, i.e. the island example (e.g. Campbell, Mitchell and Reece, 2000). There is no genetic flow between the island and the mainland; subsequently, populations on the island diverge from those of the same species on the mainland and other islands. Geographic isolation need not be this extreme, and in fact geographic isolation very much depends on the mobility of the organism. For example, two populations of birds separated by over a hundred kilometres may have a good chance of interbreeding with each other, and may do so relatively often (Reinhardt et al, 1997). Two populations of salamanders on the other hand, separated by the same distance, may never have a chance to exchange genetic material. This may lead to a faster divergence of the latter organisms as compared to the former.

The purpose of this study is to examine the amount of genetic similarity or relatedness (which would be negatively correlated with divergence of the individuals in question) of two populations of *D. fuscus* in Ohio. Our findings suggest that a larger geographic separation is needed; more time is needed after the separation of the populations; or that the similarity of the two sites leads to similar selective pressures, and that the populations have not undergone a large amount of differentiation from each other yet.

Description of Study Sites

We collected individuals for use in this study from Vickroy Hollow, Hocking State Forest, Hocking County, Ohio, near Conkle's Hollow; and from the campus of Denison University, located in Granville, Licking County, Ohio, behind Barclay Field (Figure 1). Both locations consisted of a stream in a wooded area. At both locations, the streams were intermittent streams, but were currently flowing when individuals were collected. The Hocking County site flows continually (but at varying levels) except during drought; the Denison University site had no reports of running dry within the past five years, at least. Leaf litter at both sites was slight at the time of collection, and soil was moist but not saturated. Both streams have sandy to pebble or bedrock bottoms, with numerous large flat rocks that would be suitable for nesting sites in the reproductive season. We initially intended to study individuals from nests that reproductive individuals would build in these areas, but due to unusual weather conditions, amendments to the study resulted in dealing with individuals only, and not nesting sites.

The Hocking County site is situated within a hemlock forest, resembling that of northern Canada. It has rich topsoil, and throughout much of the area, a relatively shallow layer of this topsoil, with streams frequently exposing the sedimentary bedrock beneath. Mushrooms are abundant, along with shade plants and ferns, as the canopy is thick and provides much protection from the drying sunlight. The area is also cooler than the surrounding area, due to the combination of shade, humidity, low altitude, and narrow, sheltering rock ravines. These factors combine to make it an ideal habitat for woodland amphibians in general, and especially salamanders, both aquatic and terrestrial.

The Denison University site lies within a deciduous forest, with multiple spring-fed and run-off streams. There is a dense canopy cover, and rich loamy soil provides ample nutrients for shade flowers in spring, as well as invasive shrubbery throughout the year. The topsoil is thick, with no exposed bedrock, and slightly pebbly. It lies near (approximately 15 metres behind, and down a small slope) an access road for the athletic field, but this is a relatively new road (less than two years since paving), and is not well maintained.

Methods

At the each of the locations, we caught individuals by hand, from under rocks and leaf litter. We recorded the spatial location of each individual upon capture, based on measurement of the distance along each stream course. At the Hocking County site, the study individuals came from near the middle of the ravine, in the 290-metre segment to the 360-metre segment of stream, as measured from the mouth of the ravine. At the Denison University site, study individuals were found in less than 12 metres of the stream, near the head of the spring. After the capture, procedures at the two sites diverge out of necessity.

Upon capturing individuals at the Hocking County site, we placed them into bags marked with the segment from which they came (along with some leaf litter for shelter and environmental enrichment, as well as replication of natural environment and lessening of stress), and chilled them to slow metabolism and further lessen stress on the individuals (Wright, 2001). This is a typical practice and poses no threat to the animals, and is possible due to the ectothermic nature of amphibians, which allows them greater flexibility of tolerable temperatures compared to other non-ectothermic animals. We then transported them back to our lab at the Ohio State University at Lima. Once there, I treated individuals with 35% ethanol for approximately 90 seconds, or until obtaining a light plane of anaesthesia, defined as the cessation of the righting reflex and gular respiration (Wright, 2001), to subdue them for manipulation and lessen their stress. Once anaesthetized, I removed approximately 10 millimetres from the tip of the tail, using flame sterilized and cooled scissors, and measured their snout-vent length. Toe clipping and tail clipping has been used on amphibians since 1947 (Bogert, 1947) by many herpetologists (Orser and Shure, 1972, 1974, Donnelly, 1989; for example), and is accepted by the

United States Geological Survey as an acceptable marking method (Green, 2001). While there is some controversy over the ethics of tissue removal (Halliday, 1994), it is generally considered safe if done properly (Reaser and Dexter, 1996), and does allow for the collection of adequate amounts of tissue for genetic and other biological assays (Mullis and Faloona, 1987; Paabo et al., 1989).

On adult-sized individuals, I assigned sex by tactile examination for pre-maxillary teeth, which protrude from the jaw of males, but are absent in females (Pfingsten, 1989). I coded those individuals with pre-maxillary teeth as male. Then, those without pre-maxillary teeth, but of adult size, were assigned female. Those lacking pre-maxillary teeth but not of adult size were recorded as unknown. Visual appearance of ova within the body cavity also served as a diagnostic feature discriminating females from males. Individuals recovered for a minimum of five minutes in tap water, after which the wound was treated with Bactine® spray and allowed to dry before being returned to their marked bag, and then the cooler. Most individuals began to recover as I finished manipulations. I avoided the possibility of heat damage to individuals by using cold water to mix with 95% ethanol to obtain the needed anaesthetic agent. Preservation of tail tips and their DNA took place in 70% ethanol, kept at room temperature and protected from light. After completing procedures, the individuals were returned to their home streams within two days.

Procedures at the Denison University site included field anaesthesia in ethanol prepared the same as the ethanol for the Hocking County site individuals. Upon capture, I performed the same manipulations as I had on the Hocking County site individuals- tail tip collections, measurements, and sexing of mature individuals. Individuals only left their location in the stream for the anaesthesia and manipulations, and returned to recover in their home location. I monitored them again after approximately five minutes to ensure their complete recovery. The ambient and stream temperature ensured the ability to avoid heat damage to the individuals.

All procedures were carried out in accordance with the Ohio State University Institutional Laboratory Animal Care and Use Committee protocol number 2004A0095, approved in June of 2004. Tail tip collection did not lead to tail autotomization in any of the individuals, nor were any of the individuals harmed in any way other than the loss of approximately ten millimetres of tail tip material. Although salamanders do store fat reserves in their tails for use when food is not available (i.e. winter), the removal of approximately ten millimetres at the tip of the tail is not expected to decrease the likelihood of survival, or increase the chance of starvation in the case of any of the individuals. In the cases of all individuals, there were no observable signs of pain or discomfort outside of the expected aversion to handling commonly present in wild animals. In an attempt to prevent infection from entering the injured area, Bactine® was applied after each animal had recovered from anaesthesia. Bactine® was the antiseptic of choice due to its analgesic properties, and the lack

of grease, oils, alcohol, or protein compounds in the formulation. It is best to avoid the use of proteins and oils on amphibian wounds due to the possibility of skin secretion disruptions by high alcohol concentrations (greater than 70% (Martin, 2001)) and the potential for regenerative complications due to the presence of foreign or alien oils and/or proteins (Balinsky, 1970).

Extraction of genetic material included whole genome extraction by phenol-chloroform-isoamyl extraction (Hillis et al., 1996A). We disrupted cellular membranes using sodium-lauryl-sulphate treatment (a detergent) (500µL sodium-Tris® EDTA buffer, 25µL proteinase K, and 75µL 10% sodium-lauryl-sulphate at 55° C for 2 hr). Separation of cellular debris from genetic material (in solution) involved three sequences of centrifugation (with 600µL phenol-chloroform-isoamyl alcohol at 7000 rpm for 5 min), pellet formation, and rinsing (once with phenol-chloroform-isoamyl alcohol, twice with chloroform-isoamyl alcohol). The transfer of supranatant liquid containing the genetic material between rounds of centrifugation ensured its preservation. The resultant liquid underwent a cycle of precipitation (45µL 2*M* sodium chloride and ~2.5mL ice-cold 190 proof ethanol, at -20° C, overnight), two rounds of centrifugation (7000 rpm for 10 min) and rinsing (with 140 proof ethanol), followed by dissolution of the resultant pellet (250µL 1X Tris® borate EDTA), to purify it and remove proteins, RNA and organelle material. Precipitation and dissolution occurred by the use of salt solutions (precipitation) and Tris-EDTA buffer (dissolution). The final solution of genetic material underwent a final dissolution in Tris borate EDTA, before storage in a freezer at approximately -20° C. All materials were mixed the day of use. I made all measurements of dry solutes on the same scale, tared daily, and kept in the same place for the duration of chemical mixing. I checked (and adjusted, if necessary) the levelling on the scale daily, before any measurements were made. All chemical usage for the purposes of DNA extraction took place over a nine-day period, and samples were stored at -20° in between procedures.

Amplification of genetic material by PCR (Palumbi, 1996) followed extraction. I slightly modified the PCR that became the basis for this procedure. The modification consisted of the addition of the long chain product (Griffiths et al., 2002) from a single round of PCR from a randomly selected individual from each of the study sites. These long chain products consisted of a single round of PCR on isolated study individuals using the original random hexamer primer from Integrated DNA Technologies, resulting in a long single stranded chain of DNA that can serve as an additional primer in subsequent PCR sequences. PCR then proceeded as described (Palumbi, 1996), using the initial random hexamer primer, and a long chain primer made from individuals from each of the study sites (solution: 2.5µL 10X *Taq* buffer, 2.5µL 8m*M* dNTPs, 1.2*µ*L each primer, 0.5-1 U *Taq* polymerase, distilled water to make 24µL, 1µL template DNA, plus 1 drop of mineral oil to prevent evaporation of sample). Thermo-cycling consisted of a 4 minute warm-up period, followed by 30

seconds at 94° C denaturation, 60 seconds at 50° C annealing, 90 seconds at 72° C elongation, and a final hold at 10°C. The denaturation, annealing, and elongation cycle was performed once for the construction of the long chain primers, and thirty-five times for the samples of DNA. The addition of the two long chain primers provides two further primer sites for analysis, as well as providing primer templates that theoretically should each be very similar to at least half of the study samples. This addition would have the net effect of increasing the results of the polymerase chain reaction (and the subsequent bands in electrophoresis) by threefold. After polymerase chain reaction, storage continued as before at approximately -20° C until gel electrophoresis.

Gel electrophoresis occurred in poly-acrylamide gels suspended in 1X Tris borate EDTA solution, at 75 V for 70 minutes (Dowling et al., 1996). Due to the chemical properties of DNA, an electrical current has the ability to pull DNA parallel to the current and toward the positive end of the gel box (the anode), dragging smaller fragments for farther distances from the starting position (Dowling et al., 1996). This allows a separation of DNA sections by fragment size, sections which then cluster with other segments of the same size at certain points, making the characteristic bands after staining (Dowling et al., 1996). The construction of the gels followed directions provided with the acrylamide/bis-acrylamide solution from Sigma chemicals. I used a gel solution of 8% (9.8mL distilled water, 4.0mL 40% acrylamide/bis-acrylamide solution, 5.0mL pH 8.9 Trizma base/TEMED solution, 0.2mL 10% sodium-lauryl-sulphate, and 1.0mL 11% ammonium persulfate solution), of approximately five millimetres depth. An 8% concentration of acrylamide solution was used for its ability to separate reasonable lengths of DNA (it can separate DNA fragments that differ by 60-400 base pairs (Sigma, 1998), and the simplest of the three primers (the random hexamer) has a chance of occurring once every 4096 base pairs (or 4^6)). This is expected to provide an adequate degree of resolution, as well as being more economical than a higher concentration. While running the gels, I also ran bromophenol blue and xylene cyanol dyes, to determine the relative migration of the DNA fragments at any given time. These dyes are known to migrate at the same velocity as DNA fragments of lengths 425 base pairs and 765 base pairs, respectively (Sigma, 1998). This allowed a rough estimation of the size of the smallest band, and ensured that DNA bands were not run off the ends of the acrylamide gel beds during electrophoresis. I made acrylamide forms using inert acrylic plastic from Home Depot, washed with ethanol, and rinsed with distilled water, and then sealed with inert silicon sealant and dried for at least 48 hours before use. I then cut out wells approximately two millimetres by one and a half millimetres by two millimetres (length by height by depth), eight across, using a new, sterile, Exacto®-type knife. DNA samples were randomized prior to placement with finished acrylamide gels by the use of a random number table (going in order of capture, also the order in which

data were entered for statistical analysis, numbers were chosen to correspond to the acrylamide gel sample well number) to simulate a blind procedure. The purpose of this was to eliminate any potential for observer bias when analyzing the DNA bands at viewing. These numbers and their corresponding animal identification number were recorded, and then everything was set aside until the next day, when the randomized samples were run. The animal identification numbers and sample well numbers were both entered into the data analysis program (SPSS version 11.5 for Windows), although only the animal identification number was used for the actual analysis. Finished, eletrophoresed gels were treated with ethidium bromide to verify DNA position within the gel.

After gel electrophoresis, the gels were subjected to a Southern blot analysis (Dowling et al., 1996; Southern, 1975), onto nitrocellulose membranes with a pore size of 0.45µm. The Southern blot technique is a method of transferring DNA from an agarose or acrylamide gel onto another, less fragile, more permanent, medium. The transfer is achieved by stacking the receiving membrane on top of the target gel, with blotter paper or paper towels placed on top of the membrane. This entire pile is then placed (slightly elevated, but still touching the solvent) onto a small pedestal covered with absorbent material (such as more blotter paper or paper towel) in a dish of 1X Tris borate EDTA. The Tris borate EDTA flows through the wick material, and up the gel, membrane, and blotter, and then evaporates. The capillary action of the material and the solvent keep the Tris borate EDTA flowing. The DNA moves along with Tris borate EDTA through the gel, but is stopped on the membrane because of the smaller pore size in the membrane compared to the gel. Thus, an exact copy of the original gel is made on a material that is more durable than the gel itself (usually nitrocellulose or 6, 6-nylon) (modification of Dowling et al., 1996).

The gels were then moved to a denaturing solution (0.4 *M* sodium hydroxide for 30 minutes), followed by rinsing with distilled water and neutralization (3 *M* sodium chloride, pH 7.5 Tris-hydrochloride for 30 minutes) (Dowling et al., 1996). The denaturation produced single stranded DNA that could then receive a single stranded, marked probe DNA piece. The neutralization allowed for the relaxing of the DNA (without re-annealing to itself), lessened the chances of irritation to the skin of those working with the membranes, and increases the expected life span of the membranes. DNA was fixed to nitrocellulose membrane by baking at 60° C for 75 minutes after drying. Membranes were then re-hybridized with a single-stranded, fluorescent marked DNA probe from Integrated DNA Technologies, Incorporated. The marker was a sequence of 5'-6-FAM ACT GAC TGA CTG ACT GAC TGA CTG -3', labelled with 6-FAM™ (6-carboxyflourscein). This molecule becomes protonated, and as a result, has decreased fluorescence below pH 7.0, and thus is typically used between pH 7.5-8.0, and has max absorbance at 495nm and 520 nm (IDT catalogue). Re-hybridization

took place in a solution of 75µL of STE-SDS (10m*M* Tris-hydrochloride, 1m*M* EDTA, 100m*M* sodium chloride, and 0.2% sodium-lauryl-sulphate), 600µL Tris-EDTA, and 25µL probe per membrane, overnight, at room temperature, in a covered inert polypropylene container. Resultant membranes were then air-dried and viewed under fluorescent light, in a darkened room (Dowling et al., 1996). This procedure was then repeated with a different marker (5'-6-FAM-GAT CGA TCG ATC GAT-3') to provide another level of analysis.

Results

Of the forty-two individuals surveyed for this project, thirty were from the Hocking County site and twelve from Denison. There were thirteen males, two from Denison (4.8% of total individuals) and eleven (26.2%) from Hocking County. There were a total of sixteen individuals of unknown sex, two from Denison (7.1%) and fourteen (33.3%) from Hocking County. There were also thirteen females, seven from Denison (16.7%) and six (11.9%) from Hocking County.

Twenty-four bands (two of which were identical to each other and so counted as the same band, but all others being unique) occurred after viewing with ethidium bromide, and the two different markers (Appendices A (Marker 1) and B (Marker 2)). Of these, band 11 was the most help as a diagnostic tool; it occurred in none of the individuals from Denison, and 90% (27 of 30) of the individuals from Hocking County (Table 1).

Statistical analysis included repeated measures *ANOVA*, with the bands as the various trials, viewing techniques as the condition, and the site as the independent variable. Analysis was performed using the computer software program SPSS for Windows (version 11.5), and showed multiple statistical differences. The data were entered with the three different techniques listed as three different conditions, and then scored as either present or not present, based on whether or not there was a visible DNA band at a particular locus. The bands were numbered from anode to cathode. Not all bands occurred in all individuals, so that there were gaps at some points for each individual. This is to be expected from a DNA method that has as its goal to pick out differences among individuals. The banding pattern seen on each individual is expected to show differences from those of other animals, with the degree of similarity correlating with the degree of relatedness. These gaps in the banding patterns were scored as a '0' or not present in the data analysis across the fragment lengths in which they occurred. The fragments lengths that did occur were scored as a '1' or present.

In the t-test for equality of means comparing bands as a function of site, there were significant differences in bands number 5, 6, and 11 (Table 1), assuming equal variances. In Levene's test for equality of variances, there were significant differences seen in band numbers 3, 6, 9, 11, 12, and 19 (Table 1). In the repeated measures *ANOVA*, using the site as the grouping variable, the visualization technique as the covariate, and the bands as the various trials, there were significant differences seen in all bands (Table 1) between

groups. Overall, for the repeated measures *ANOVA*, the significance of the band pattern was high (p= .002), as was the band-site interaction (p= .000). As a test of within subjects effects, the banding pattern was significant (p= .015), as well as the band-site interaction (p= .000).

Discussion

The purpose of this study is to examine the extent of genetic differentiation (which would be negatively correlated with percent of shared genetic characteristics of the individuals in question) of two populations of *D. fuscus* in Ohio. Our findings suggest that a larger geographic separation; more time after the separation of the populations; or a larger dissimilarity between the two sites may be needed before the two populations can undergo a larger degree of genetic differentiation from each other.

The differences noted in this study are not nearly as great as those found by Tilley and Mahoney (1996), who found species-level differences within a few miles of each other, but they do indicate significant genetic differences among populations of salamanders. This is generally what had been expected, but was not as pronounced an effect as we would have liked. There are many explanations for the decreased level of genetic differentiation seen in the Tilley study compared to this one, not the least of which is the choice of markers and the species being investigated. Tilley, Verrell, and Arnold (1990) used allozymes, as opposed to genetic markers, which may be subject to post-production alteration, and more prone to multiple gene interaction, making allozymes potentially more volatile than genetic markers, such as those use in this study. Conversely, the markers employed in this study may simply be more conservative than those used in the Tilley studies. Whereas the many genes potentially involved in allozyme production may be subject to higher gross mutation rate (due to the large number of potential mutations across a larger number of genetic sequences), the relatively small sequences used in this study (Marker 1 with 24 base pairs and Marker 2 with 18 base pairs) may simply be less likely to mutate, as the rate of mutation is functioning on a smaller fraction of the genome in this study than in those of Tilley and colleagues.

Other possibilities include a higher degree of mobility than originally suspected in this species, or convergent allozyme evolution (with many gene(s) or combinations leading to the same final product), or divergent allozyme evolution (with small changes in the ancestral genetic code leading to the same allozyme currently, but the changes being detectable at a later date). More work is needed before any conclusions as to the actions of evolution, speciation, and hybridization can be made.

Also to be considered are the differences in habitats between the Southern Appalachians (studies of Tilley and colleagues) and Ohio (this study). The Appalachian Mountains have a broad range of habitat both across distances and up and down mountainsides. This creates a larger spectrum of possibilities for

specialization and adaptation compared to the relatively homogeneous habitat found in Ohio. The Appalachian Mountains also may provide better habitat for amphibians, which has been available for a longer time, as compared to Ohio habitats. This could allow larger numbers of individuals to survive, thus increasing the gene pool and subsequently, the variability of the population. This increase in the variability of individuals would allow greater room for selective pressure that is present to work upon, possibly leading to a greater potential for divergence between populations.

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Captions

Figure 1. Map of Ohio, with study sites (Vickroy Hollow-1; Denison-2) marked, and glacial boundary shown (Vickroy Hollow, unglaciated; Denison University, glaciated). Modified from Peterjohn and Rice, 1991.

Appendix B

Table of Statistics of Import

	Significance						
Band	Equality of means	Equality of variances	Repeated Meas. *ANOVA*	Frequency of Markers (%)			
1	.658	.518	<.000***	30	33	33	33

2	.750	.488	<.000***	30	40	25	33
3	.261	.016*	<.000***	23	40	17	25
4	.275	.604	<.000***	20	37	50	42
5	.026*	.519	.001**	37	20	50	42
6	.044*	<.000***	<.000***	30	37	42	75
7	.140	.784	<.000***	17	40	42	50
8	.691	.469	<.000***	27	40	42	42
9	.063	.001**	<.000***	23	43	0	100
10	.202	.201	<.000***	40	40	42	17
11	<.000***	<.000***	.003**	43	47	0	0
12	.074	<.000***	<.000***	33	40	58	75
13	.780	.559	<.000***	37	47	42	50
14	.658	.518	<.000***	30	47	33	33
15	.649	.491	<.000***	30	37	42	25
16	.330	.743	<.000***	37	40	25	25
17	.313	.488	<.000***	37	40	25	33
18	.979	.958	<.000***	37	43	33	42
19	.113	.007**	<.000***	30	37	50	42
20	.821	.666	<.000***	47	33	33	33
21	.608	.543	<.000***	37	33	42	33
22	.986	.971	<.000***	33	37	33	33
23	.805	.971	<.000***	43	37	42	33
Marker				1	2	1	2
				Hocking			Denison

* - significant at the ά = .05 level
** - significant at the ά = .01 level
*** - significant at the ά < .000 level

Appendix A

Table of Individuals with Marker 1 at Each Band and Frequency of Marker 1 among Populations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	2
2F01		1	1	1	1	1	1			1		1	1		1		1	1		
2F02	1	1	1		1		1		1		1	1	1	1	1	1			1	1
AM01	1			1		1	1	1		1	1						1			
2F03							1	1	1					1		1	1	1	1	
AF01						1														
2M01		1								1		1	1			1		1	1	1
2F04		1	1			1		1					1							
2M02			1			1		1	1		1		1	1					1	
AM02			1			1		1	1		1		1			1				
2F05			1	1			1					1		1						1
AF02	1		1	1		1			1		1		1		1		1	1		
AF03	1	1			1							1	1			1				
AM03	1			1		1		1		1	1		1			1	1	1		
AM04	1	1				1				1		1		1	1	1		1		1
AF04	1	1		1						1				1	1					
2M03									1	1	1	1	1	1	1		1		1	1
2F06			1			1					1			1		1		1	1	
2F07		1	1					1	1			1		1			1	1		
2M04		1				1	1	1	1	1					1	1		1	1	
0F01				1			1										1			
1F01	1	1		1			1		1		1	1	1		1	1		1	1	1
2F08			1				1	1	1		1				1	1		1		1
2F02	1						1		1		1		1	1				1		
2F09				1				1		1	1			1			1			
2F10					1		1		1	1					1				1	
AM05		1		1				1		1		1	1	1			1		1	
2F11											1	1			1					1
AF05			1		1			1		1	1	1	1	1			1			
0F03					1				1							1				1
AM06	1	1	1	1			1							1			1	1	1	1
DF01			1	1	1		1		1			1	1				1			
DF02					1	1		1	1			1	1							1
DF03		1		1		1			1			1	1	1						
DM01	1	1		1		1		1	1			1			1	1	1			
DF04	1	1		1	1	1	1		1	1								1	1	
DF05		1			1	1			1	1		1		1	1	1				
D01	1		1			1			1			1	1	1	1	1		1	1	1
DF06	1		1				1	1	1			1					1			1
DF07						1	1	1	1				1					1	1	
DM02				1		1	1	1	1			1					1	1	1	
D02					1		1		1			1	1							1
D03						1			1					1				1	1	
HSF %	0.33	0.4	0.4	0.37	0.2	0.37	0.4	0.4	0.43	0.4	0.47	0.4	0.47	0.47	0.37	0.4	0.4	0.43	0.37	0.
DEN %	0.33	0.33	0.25	0.42	0.42	0.75	0.5	0.42	1	0.17	0	0.75	0.5	0.33	0.25	0.25	0.33	0.42	0.42	0.

HSF= Hocking State Forest (individuals 2F01-AM06)
DEN= Denison (individuals DF01-D03)

Appendix B

Table of Individuals with Marker 2 at Each Band and Frequency of Marker 2 among Populations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2F01					1	1					1	1	1		1		1		
2F02	1			1						1									
AM01				1					1	1	1			1		1		1	1
2F03			1		1	1		1	1				1	1				1	1
AF01	1	1			1	1	1	1			1	1			1				
2M01	1	1									1						1		
2F04	1	1	1								1					1			
2M02		1	1			1													1
AM02														1		1		1	
2F05							1		1			1		1					
AF02	1				1				1	1	1		1		1		1		
AF03					1					1			1			1			
AM03						1		1			1	1	1		1	1		1	
AM04		1						1						1			1	1	
AF04	1	1			1		1			1			1			1		1	1
2M03		1		1						1		1							
2F06			1						1				1			1	1		
2F07						1		1			1			1			1		1
2M04			1									1		1	1				
0F01				1	1	1						1							1
1F01		1				1					1	1	1			1		1	
2F08					1	1		1										1	
2F02	1						1			1	1			1	1			1	
2F09				1						1			1		1		1		
2F10	1			1					1	1	1								
AM05	1						1			1			1			1	1	1	1
2F11					1							1				1			1
AF05					1						1		1	1	1	1	1		1
0F03		1	1		1			1		1	1	1			1		1		
AM06			1					1	1	1							1	1	
DF01	1	1		1	1			1		1		1		1	1				
DF02				1			1	1				1		1					
DF03	1			1		1	1					1	1		1				1
DM01				1		1	1									1	1	1	
DF04				1		1	1	1		1		1		1				1	1
DF05	1				1					1			1		1		1		

D01														1	1					
DF06					1	1								1					1	1
DF07	1		1	1	1			1				1	1		1	1				
DM02		1						1		1		1					1			1
D02		1	1		1	1				1		1			1					1
D03					1		1									1			1	1
HSF %	0.3	0.3	0.23	0.2	0.37	0.3	0.17	0.27	0.23	0.4	0.43	0.33	0.37	0.3	0.3	0.37	0.37	0.37	0.3	
DEN %	0.33	0.25	0.17	0.5	0.5	0.42	0.42	0.42	0	0.42	0	0.58	0.42	0.33	0.42	0.25	0.25	0.33	0.5	

HSF= Hocking State Forest (individuals 2F01-AM06)
DEN= Denison (individuals DF01-D03)